WRAIR PROTOCOLS FOR SOLDIER STATUS AND READINESS TO ORGANOPHOSPHATE EXPOSURE: UNPROCESSED WHOLE BLOOD CHOLINESTERASE AND PYRIDOSTIGMINE BROMIDE QUANTIFICATION

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ABSTRACT

Exposure to nerve agents, OPs, pesticides, anesthetics, terrorists' chemical agents, and drugs of abuse such as cocaine, heroin, and some neuro-degenerative disease states selectively reduces AChEand/or BChE activity. Since urban terrorism is on the rise, Federal,State, and local authorities need a reliable, fast, inexpensive method for confirming such an assault to initiate appropriate containment, decontamination, and treatment measures. Thus, we developed a semi-automated medical diagnostic microplate procedure capable of screening unprocessed whole blood samples for the concentrations of AChE and BChE (patent pending, WRAIR) to overcome limitations of current methods. We applied our technique to whole blood in man, non-human primates, and rodents. In addition, pyridostigmine bromide (PB) treatment is the only prophylactic treatment for troops who expect to be exposed to OPs. Thus, we developed a sensitive HPLC technique to quantify PB in human blood and also in Rhesus and rodent blood and tissue. The technique uses solid phase extraction, lyophilization for concentration, and HPLC of the reconstituted samples followed by strong-cation exchange chromatography via isocratic elution. These techniques will provide the soldier, field medic, hospital, company, or
civilian organization with the ability to rapidly determine exposure to OPs and aid in treatment while the HPLC assay will aid in verifying troop compliance when required for PB prophylaxis against OPs.

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INTRODUCTION

The physiological role of acetylcholinesterase (AChE, EC 3.1.1.7) is the acceleration of hydrolysis of the neurotransmitter acetylcholine at nerve-nerve and neuromuscular junctions. This enzyme possesses one of the fastest turnover rates known. Inhibitors of AChE have been used medicinally in the treatment of glaucoma, myasthenia gravis, and recently for Alzheimer's disease with FDA approval of tacrine and Aricept. Other inhibitors have been used agriculturally and domestically as pesticides and insecticides. Unfortunately, some of the most potent inhibitors of cholinesterases have been developed as chemical warfare agents (CWA), e.g., GA (tabun), GB (sarin, the agent used by the terrorist group in Japan in 1995), GD (soman), and VX, These organophosphorus (OP) nerve agents are a serious threat to US military personnel. The sequel to AChE poisoning is a cholinergic crisis in man; the clinical effects are directly related to acetylcholine accumulation.

Exposure to nerve agents, OPs, pesticides, anesthetics, terrorists' chemical agents, drugs of abuse such as cocaine, heroin, and some neuro-degenerative disease states selectively reduces AChE and/or BChE activity. Thus, blood cholinesterase activity can be exploited as a tool for confirming exposure to such toxic agents. Furthermore, since urban terrorism is on the rise, Federal, State, and local authorities need a reliable, fast, inexpensive, and standard method for confirming such an assault in order to initiate appropriate containment, decontamination, and treatment measures. Finally, pyridostigmine bromide (PB) treatment is the only prophylactic treatment for troops who expect to be exposed to OPs. PB (30 mg tablet, twice daily recommended) results in about 30% reversible inhibition of normal levels of blood cholinesterase. Therefore, the screening method described here can also be utilized to determine the level of blood cholinesterase protected by PB and thus the protective status of troops prior to deployment.

Current clinical determination of cholinesterase levels of blood includes the Michel, microMichel, pH stat, Ellman, and microEllman methodologies. These methods, however, have long clinical turn around times, lack of standardization among labs, and normally determine only the serum or red blood cell cholinesterase concentration, but not both. In addition to the clinical methodologies, a field deployable unit is commercially available: the Test-Mate OP™ system. The kit requires several processing steps and the use of a selective BChE inhibitor for complete AChE and BChE screening.To overcome these limitations of current methods, we developed a semi-automated medical diagnostic microplate procedure capable of screening unprocessed whole blood samples for the concentrations of AChE and BChE at unprecedented precision (<1 %) and accuracy (>99%) (patent pending, WRAIR). In addition, the procedure is being ported to a high-throughput robotic system. We have successfully applied our technique to whole blood in the presence and absence of selective (huperzine-a and Iso-OMPA), and non-selective (pyridostigmine bromide) cholinesterase inhibitors. This is possible because blood contains two cholinesterases that possess different affinities for any given substrate, and a linear correlation exists between enzyme activity and concentration. Thus, if one determines the activity of any given blood sample with two different substrates, then it is feasible to calculate the precise concentrations of both proteins (i.e., two equations with two unknowns). Furthermore, monitoring the activity with three substrates (Figure 1) provides three fold degenerate data [i.e., three sets of two equations with two unknowns]. The rates of substrate hydrolysis represented by R_1, R_2, and R_3 correspond to the turnover of substrate 1, substrate 2, and substrate 3, respectively. [AChE] and [BChE] refer to the actual concentrations of AChE and BChE contained in the sample. Finally, the coefficients in each equation (i.e., x_1, x_2, x_3, and y_1, y_2, y_3) represent the sensitivity coefficients. These coefficients represent the contribution that AChE and BChE independently contribute to the overall rate of substrate hydrolysis (R_1, R_2, R_3). Simultaneously solving these three sets of degenerate equations provides three independent estimates for the concentrations of AChE and BChE. Therefore, determining the mean value and the standard deviation for these independently derived values provides an excellent estimate of the authentic concentrations of each protein.

$$x_1[\text{AChE}] + y_1[\text{BChE}] = R_1 \quad \text{Substrate 1}$$
$$x_2[\text{AChE}] + y_2[\text{BChE}] = R_2 \quad \text{Substrate 2}$$
$$x_3[\text{AChE}] + y_3[\text{BChE}] = R_3 \quad \text{Substrate 3}$$

$$[\text{AChE}]_{1,2} = \frac{\begin{vmatrix} x_1 & R_1 \\ x_2 & R_2 \end{vmatrix}}{\begin{vmatrix} x_1 & y_1 \\ x_2 & y_2 \end{vmatrix}} \quad \text{and} \quad [\text{AChE}]_{1,3} = \frac{\begin{vmatrix} x_1 & R_1 \\ x_3 & R_3 \end{vmatrix}}{\begin{vmatrix} x_1 & y_1 \\ x_3 & y_3 \end{vmatrix}} \quad \text{and} \quad [\text{AChE}]_{2,3} = \frac{\begin{vmatrix} x_2 & R_2 \\ x_3 & R_3 \end{vmatrix}}{\begin{vmatrix} x_2 & y_2 \\ x_3 & y_3 \end{vmatrix}}$$

$$\therefore \text{mean } [\text{AChE}] = \frac{[\text{AChE}]_{1,2} + [\text{AChE}]_{1,3} + [\text{AChE}]_{2,3}}{3}$$

The [BChE] can be calculated in a similar manner.

Figure 1. Mathematical representation of the WRAIR sensitivity coefficient method.

We found that the results obtained from these experiments parallel those in the literature. In addition, the sensitivity coefficient method has been applied to several species including human, rhesus monkey, Hartley guinea pig, and Sprague Dawley rat. Thus, blood levels of cholinesterases after exposure to CWAs such as GD and pharmaceuticals such as huperzine-a and pyridostigmine have been determined in animals and man. We have also performed comparisons with the COBAS/FARA and TestMate OP™ systems, which yielded identical trends. However, unlike the conventional clinical tests or the Test-Mate OP™ unit, our method provides a full analysis of the patient's cholinesterase levels, does not rely on the addition of selective AChE or BChE inhibitors, uses a single non-invasive blood collection technique (currently a finger prick), is not labor intensive, and produces results in less than six minutes.

A field unit for blood cholinesterase (i.e., individual use) is in development; it is based on the new technology of a single use disposable silicon needle (US patent #5,801,057). This needle is minimally invasive and painless, removes less than a drop of blood, and is now being commercialized for diabetic blood glucose monitoring. Detection for measuring the rate of cholinesterase-catalyzed hydrolysis has been demonstrated with a single substrate redox-mediated electrochemical sensor utilizing butyrylthiocholine as a substrate, which is hydrolyzed by the enzyme to form thiocholine. The latter product is consumed by reacting with ferricyanide, and is then oxidized at the electrode to form ferrocyanide. Within 60 sec after initiation of the assay, a level of cholinesterase reduced more than 50% is readily observed by the electrochemical detector. The hollow silicon microneedle, comparable in cross-section to a human hair, is now being integrated with lab-on-a-chip microfluidics.

Lastly, to verify compliance of ordered PB consumption (when expecting OP exposure), we have developed a sensitive HPLC technique to quantify PB in human blood and also in Rhesus, and rodent blood and tissue. This is an alternate and confirmatory technique for measurement of PB levels directly to supplement the indirect assay by inhibition of ChEs. The PB HPLC technique is based on solid phase extraction, lyophilization for concentration, and HPLC of the reconstituted samples using strong-cation

exchange chromatography and isocratic elution. The linear dynamic range of sensitivity covers at least 500 to 0.00125 μg of PB. This assay lends itself to high-throughput and direct PB quantification. The utility and robustness of the technology is shown by adoption at other DOD agencies involving measurements of PB in plasma samples and it is used in studies at the Uniform Services University of Health Sciences for low-dose combination effects of PB, atropine, and pyrimethamine in Gulf War syndrome investigations.

While the cholinesterase assay method using a handheld device is designed for the 1st echelon (or by first responders), the high-throughput assay is readily performed at 3rd/4th echelons (and in civilian clinical laboratories). These techniques will provide the soldier, field medic, hospital, company, or civilian organization with the ability to rapidly determine exposure to OPs and aid in treatment. In addition, the sensitive HPLC assay for quantifying PB levels will aid in verifying troop compliance when required for prophylaxis against OPs.